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Production of mannitol by *Lactobacillus intermedius* NRRL B-3693 in fed-batch and continuous cell-recycle fermentations

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Abstract

Improved fermentation processes were developed for the production of mannitol by a heterofermentative lactic acid bacterium (*Lactobacillus intermedius* NRRL B-3693). A fed-batch fermentation protocol overcame limitations caused by high substrate concentrations. The process was developed using corn steep liquor and glucose as inexpensive industrial nutrient sources, supplemented with a small amount of soy peptone and manganese. The fed-batch process resulted in a concentration of 176 ± 0.5 g mannitol from 184 ± 0 g fructose and 92 ± 0.1 g glucose per L of final fermentation broth in 30 h with a volumetric productivity of 5.9 g/(L h). Further increases in volumetric productivity of mannitol were obtained in a continuous cell-recycle fermentation process that reached more than 40 g/(L h), despite reduced mannitol levels of 78–98 g/L and residual substrate of 10–20 g/L. This is the first report of such a high volumetric productivity of mannitol by a heterofermentative lactic acid bacterium.

Keywords: Mannitol production; *Lactobacillus intermedius*; pH-controlled fermentation; Fed-batch fermentation; Continuous cell-recycle fermentation; Lactic acid bacterium

1. Introduction

Mannitol, a naturally occurring polyol, is widely used in the food, pharmaceutical, medicine, and chemical industries [1]. It (U.S. $3.32/lb; global market, 30 million lbs/year) is currently produced industrially by high-pressure hydrogenation of fructose/glucose mixture in aqueous solution at high temperature (120–160 °C) with Raney nickel as a catalyst [2]. Typically, the hydrogenation of a 50/50 fructose/glucose mixture results in an approximately 25/75 mixture of mannitol and sorbitol ($0.73/lb). This means that about half of the fructose is converted to mannitol and half of it to sorbitol. The glucose is hydrogenated exclusively to sorbitol. As a consequence, the commercial production of mannitol is always accompanied by the production of sorbitol thus resulting in an inefficient process [3]. Moreover, it is relatively difficult to separate sorbitol and mannitol, which results in even higher production costs and decreased yields [4]. Some microorganisms can specifically produce mannitol from glucose or fructose without making sorbitol [5–7]. Thus, research efforts have been directed toward production of mannitol by fermentation and enzymatic means [8].

Several heterofermentative lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Leuconostoc*, and *Oenococcus* produce mannitol from fructose [9–12]. In our previous paper, we reported the production of mannitol by *Lactobacillus intermedius* NRRL B-3693 from fructose using a simplified MRS medium [13]. The fermentation method makes use of the capability of the bacterium to utilize fructose as an alternative electron acceptor, reducing it to mannitol with the enzyme mannitol dehydrogenase (EC 1.1.1.67) [14]. The reducing equivalents are generated by conversion of about one-third of the fructose to lactic acid and acetic acid. The *L. intermedius* NRRL B-3693 strain can convert fructose completely to mannitol from a mixture of glucose and fructose (1:2). Glucose is converted to lactic acid and acetic acid, and the fructose is converted to mannitol. Mannitol, at 180 g/L concentration, can be easily recovered from the fermentation broth by cooling crystallization [13]. However, achieving such a high mannitol concentration in a

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batch process requires high concentrations of fructose which results in long lags in production. Fed-batch fermentation by adding substrate at three times decreased the time of maximum mannitol production from fructose (300 g/L) from 132 h to 92 h [13]. Bacto-peptone (Difco Laboratories, Detroit, MI, $59.95/lb) and Bacto-yeast extract (Difco, $71.50/lb) are the most expensive components of the simplified MRS medium. In order to produce mannitol cost-effectively on an industrial scale by fermentation, more economical nutrient sources are required to replace the expensive Bacto-peptone and Bacto-yeast extract. Most of the methods reported for mannitol production by LAB use expensive peptone and yeast extract [7,15]. We have been able to simplify the components of the MRS medium and have shown that high levels of mannitol productivity could be attained using soy peptone, corn steep liquor, and manganese sulfate in batch fermentation processes [16–18]. We have further extended these results and developed a fed-batch fermentation process for this LAB that attained high final mannitol concentration in the fermentation broth without the lag period, resulting in higher volumetric productivity. The highest volumetric productivities for many fermentation processes are typically obtained using cell-recycle fermentation protocols [19–21]. In this paper, we demonstrate that mannitol production by *L. intermedius* NRRL B-3693 responds very well to this type of process, reaching volumetric productivities of greater than 40 g/(L h).

2. Materials and methods

2.1. Materials

High fructose corn syrup (HFCS, IsoSweet® 5500, 55% fructose–41% glucose, 77% solids) and Krystar® liquid fructose (99.5% fructose, 77% solids) were products of Tate and Lyle, Decatur, IL. Corn steep liquor was supplied by Cargill, Minneapolis, MN. The general composition of corn steep liquor is carbohydrates, amino acids, polypeptides, fatty acids and other organic compounds, hydrolytic enzymes, heavy metals, and inorganic ions as reported by Hull et al. [22]. Soy peptone HSP-A was obtained from Nutricepts, Inc., Burnsville, MN. Protease GC106 was from Genencor International, Rochester, NY. Aminex HPX-87P (300 mm × 7.8 mm) and Carbo P micro-guard cartridge (30 mm × 4.6 mm) were purchased from Bio-Rad Laboratories, Hercules, CA. Hollow fiber filter (66.7 cm × 1.9 cm; membrane area, 0.085 m^2; lumen diameter, 1 mm; Model UFP-500-E-4X2MA) was purchased from A/G Technology Corp., Needham, MA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

2.2. Bacterial strain

L. intermedius NRRL B-3693 was obtained from the ARS Culture Collection (National Center for Agricultural Utilization Research, Peoria, IL). Stock cultures were maintained in 75% glycerol at −70 °C. Cultures were transferred in agar (15 g/L) slants made with simplified MRS medium containing 10 g peptone, 5 g yeast extract, 2 g ammonium citrate, 100 mg magnesium sulfate, 50 mg manganese sulfate, and 2 g disodium phosphate per L (final pH 6.5) [23]. Glucose (10 g/L) was used as the energy source. After growth at 37 °C overnight, slants were stored at 4 °C for use in seed culture preparation.

2.3. Preparation of seed culture

The simplified MRS medium described above was used for the preparation of the seed culture. The medium and the substrate were sterilized separately at 121 °C for 15 min. A 250-mL Erlenmeyer flask containing 100 mL of the medium with fructose (20 g/L) was inoculated with a loopful of cells taken from a stock slant and incubated at 37 °C on a rotary shaker at 130 rpm for 24 h. This culture was used as the seed culture.

2.4. Fermentation experiments

Fed-batch fermentations were conducted in 2 L fermenters (Biostat® B, B. Braun Biotechnology International, Allentown, PA). Glucose and fructose solutions were prepared by mixing appropriate amounts of HFCS and liquid fructose to obtain the indicated concentrations. The fed-batch fermentation medium contained 112 g corn steep liquor, 80 mg manganese sulfate monohydrate, and 3.5 g soy peptone in 830 mL water which was sterilized in the fermenter at 121 °C for 15 min. A solution of 67 g fructose plus 33.5 g glucose in 120 mL water was sterilized separately and added to the fermenter when cooled. Agitation was applied at a rate just sufficient to mix the pH control reagent and feed but minimize mixing air into the medium (130 rpm). No sparge air or other gas was applied. Filter-sterilized protease (0.4 mL) was then added, the temperature was adjusted to 37 °C, and the pH was adjusted to 5.0 with 5N NaOH. The fermenter was inoculated with 50 mL of seed culture and automatic pH control at pH 5.0 with 5N NaOH was initiated. Additional substrate (400 mL) containing 228 g fructose and 114 g glucose was added at a rate of 22 mL/h beginning 2 h after inoculation.

For cell-recycling, fermentations were conducted in a batch mode for 14 h to build cell mass. Fermenters with a 1 L working volume were prepared initially with 120 g fructose and 60 g glucose (added as 200 g of high fructose corn syrup and 54 g of Krystar® liquid fructose concentrate) and 60 g corn steep liquor per L. A hollow fiber filter was sterilized with 0.2N NaOH and rinsed with sterile water. After incubation of the fermenters for 14 h, when the initial substrate was exhausted, a continuous feed was started. The feed solutions contained 100–160 g/L fructose as indicated and glucose equal to 50% of the fructose. The feed solutions also contained manganese sulfate (50 mg/L) and corn steep liquor (5–30 g/L). Feed was added to the fermenters at various dilution rates (0.15–0.55 h^{-1}) as indicated. At the same time, broth from the fermenter was pumped through the sterilized hollow fiber filter at 500 mL/min and returned to the fermenter. The inlet pressure on the filter was maintained in the 4–7 psi range. Permeate flow from the filter was controlled by a pump on the permeate outlet. Permeate was removed at the same rate as the addition of feed plus base in order to keep a constant broth volume in the fermenter. A series of fermentations, each lasting 48–96 h, were run at various substrate concentrations, dilution rates, and corn steep liquor concentrations.

2.5. Analytical methods

Sugar utilization and product analysis were performed using high-pressure liquid chromatography (HPLC) (Thermo Separation Products, Inc., San Jose, CA). An Aminex HPX-87P column with a Carbo-P micro-guard column was used. The column was maintained at 85 °C, and the sugars and mannitol were eluted with deionized water (Milli-Q, Millipore Corp., Bedford, MA) at a flow rate of 0.6 mL/min. Fructose, glucose, and mannitol were detected by refractive index and identified and quantified by comparison to retention times of authentic standards. Cell growth was measured as optical density at 660 nm. To determine cell dry weight (cdw), cells of known optical density were pelleted from a 10 mL sample at 12,000 × *g* for 10 min and washed once with water under the same conditions. The resulting pellets were dried under vacuum at 80 °C for 22 h and weighed. A converion factor of 3.09 ± 0.05 OD per g cdw was determined and used to estimate the cell mass from optical density at 660 nm.

3. Results and discussion

3.1. Fed-batch fermentation

The fed-batch process described above gave very good mannitol production rate and also final concentration of mannitol. This is evident from the results of the fermentation presented in Fig. 1. To reduce raw material cost, we replaced one-third of the fructose with less expensive glucose [13]. A fed-batch approach with initial lower concentrations of total sugars

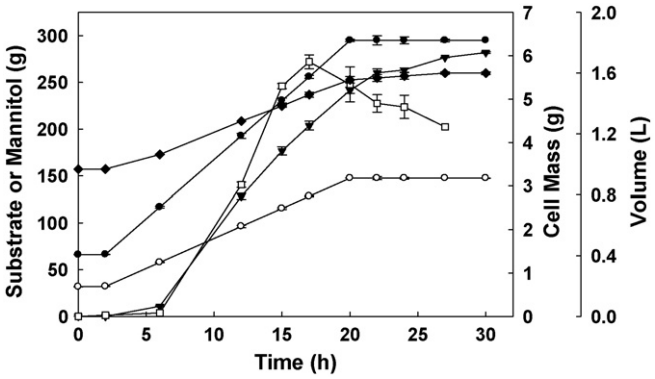

Fig. 1. Time course of mannitol production by *Lactobacillus intermedius* NRRL B-3693 in pH-controlled fed-batch fermentation at 37 °C, pH 5.0, and 130 rpm. Values reported are averages from three separate experiments. Symbols: (●), fructose added; (○), glucose added; (▼), mannitol produced; (□), cell mass; (◆), volume of fermentation broth.

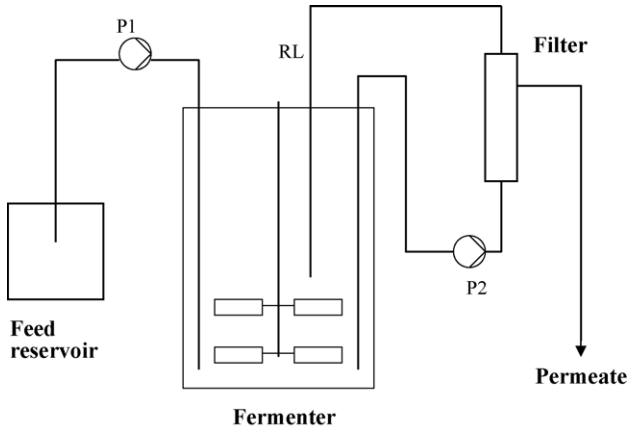

Fig. 2. Diagram of the cell recycle system. A peristaltic pump (P1) adds fresh feed solution to the fermenter while a second pump (P2) pumps the fermenter broth into the hollow fiber filter. Cells return to the fermenter through the return line (RL) while permeate is withdrawn into a reservoir.

(100.5 g/L; fructose, 67 g and glucose 33.5 g per L) was used to reduce the lag phase seen at high fructose level (300 g/L). In this case, fructose and glucose concentrations were maintained at a combined level of less than 110 g/L. Initial volume of the fermentation medium was 1 L which became 1.6 L after feeding of 400 mL substrate and adding 5 M NaOH required for maintaining the pH of the broth at 5.0 during the entire fermentation period. Final mannitol accumulation reached 176 ± 0.5 g/L from a total of 184 ± 0 g/L fructose and 92 ± 0.1 g/L glucose per L of final fermentation broth in 30 h, at a volumetric productivity rate of 5.9 g/(L h). This is the shortest reported fermentation producing such a high concentration of mannitol. Also, it was done using soy peptone (2.5 g/L), corn steep liquor (80 g/L), and manganese sulfate (50 mg/L). The cell mass of the fermentation reached a maximum of 4.02 ± 0.16 g dcw/L at 17 h, then declined slowly over the following 10 h. The specific mannitol productivity ranged from 2.04 g/(g dcw h) at 17 h to 2.35 g/(g dcw h) at 27 h.

3.2. Continuous cell-recycle fermentation

Continuous cell-recycle fermentation processes have been employed to achieve high cell densities and volumetric productivities [19–21]. We conducted a series of 1 L fermentations to examine mannitol production by *L. intermedius* NRRL B-3693 in continuous cell recycle fermentations. Two major variables were tested: dilution rate and concentration of corn steep liquor in the feed. A schematic diagram of the cell recycle fermentation system is shown in Fig. 2. Cell recycle was achieved with a hollow fiber microfilter. The most significant variable was found to be the dilution rate (Fig. 3). Over a range of dilutions from 0.15 h^{-1} to 0.55 h^{-1}, volumetric mannitol productivity was directly proportional to the dilution rate and a range of 15–40 g/(L h) was obtained. Therefore, it is possible to increase the volumetric productivity by more than five times in continuous cell-recycle fermentation than the fed-batch process described earlier. In contrast, the mannitol concentration in the permeate did not vary in proportion to the dilution rate. The R^2 values for the trendline for mannitol concentration versus dilution rate was only 0.1408 compared to 0.9117 for productivity. The mannitol concentration in the permeate ranged from 78 g/L to 98 g/L or at best about 50% of the maximum concentration of the fed-batch process. Cell densities ($A_{660\ nm}$) increased during the cell recycle phase, typically from about 15 at the end of batch growth to a maximum of 55–60. Although the initial rates of cell mass increase correlated with dilution rate, the final cell densities were similar for all dilutions. It should be emphasized that the results are the average mannitol production rates over a period of time at the indicated dilution rates and not instantaneous production rates and that cell densities were changing during these periods. Therefore, specific mannitol productivity was difficult to assess for these experiments. However, to compare the specific mannitol productivity of the continuous process

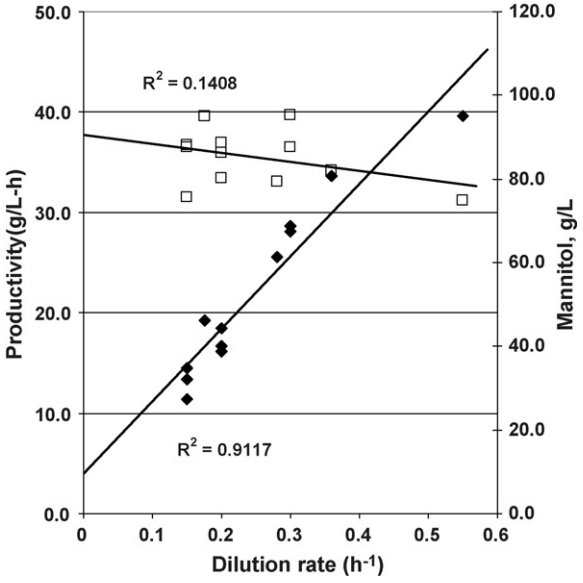

Fig. 3. Effect of dilution rate on volumetric productivity and concentration of mannitol by *L. intermedius* NRRL B-3693 in pH-controlled cell recycle fermentations at 37 °C, pH 5.0, and 130 rpm. The R^2 values for the trendlines were: 0.9117 for volumetric productivity and 0.1408 for mannitol concentration. Symbols: (□) mannitol concentration; (●) volumetric productivity.

Table 1
Summary of mannitol productivity by batch, fed-batch and continuous fermentation

Mode	Time (h)	Cell mass (g/L)	Mannitol (g/L)	Specific mannitol productivity (g/(g dcw h))	Volumetric mannitol productivity (g/(L h))	Mannitol yield (g/g)[d]
Batch[a]	136	2.7 ± 0.0	198.0 ± 4.7	0.5 ± 0.01	1.5 ± 0.03	0.95 ± 0.01
Fed-batch[b]	27	2.8 ± 0.1	172.9 ± 0.5	2.3 ± 0.04	6.4 ± 0.1	0.94 ± 0.00
Continuous[c]	27	17.0 ± 0.9	94.7 ± 0.2	1.7 ± 0.03	28.4 ± 0.4	0.94 ± 0.02

[a] Data from Ref. [13].
[b] Data from Fig. 1.
[c] Average of two fermentations of 1 L volume with a dilution rate of 0.3 h^{-1} yielding 8.1 L of permeate containing 766.8 g mannitol in 27 h.
[d] Yield of mannitol from fructose in a medium with a ratio of 2 parts fructose to 1 part glucose.

with batch and fed-batch results, we have calculated the average specific mannitol productivity for two experiments with a dilution rate of 0.3 h^{-1} (Table 1). The specific mannitol productivity of the continuous process in this example was intermediate between the batch and fed-batch processes. This lower value for the continuous process versus the fed-batch probably could reflect some accumulation of dead cells rather than a true change in specific productivity. Our value for continuous specific productivity was close to those reported by van Weymarn for mannitol production by *L. mesenteroides* at similar cell densities in a semi-continuous process [21].

Varying the fructose concentration in the feed between 90 g/L and 150 g/L did not significantly alter the mannitol concentration (data not shown). Although the exact reason for this is not known, these concentrations are probably above saturation for either the transport of fructose or the conversion by mannitol dehydrogenase, or both. Fructose and glucose were not completely consumed in this process, even at low dilutions, and residual total sugar concentrations ranged from 10 g/L to 20 g/L. As in the batch process, glucose was consumed at a rate of one-half that of fructose.

Finally, varying corn steep liquor concentration between 10 g/L and 30 g/L in the feed solution did not have much effect on the mannitol productivity but when the corn steep liquor concentration was reduced to 5 g/L, the productivity declined sharply (Fig. 4). Lower corn steep liquor concentrations are desirable to reduce the level of impurities corn steep liquor introduces into the final product. For example, the color of the permeate, as measured by absorbance at 400 nm, increased from 0.17 to 0.66 as corn steep liquor concentration increased from 5 g/L to 70 g/L.

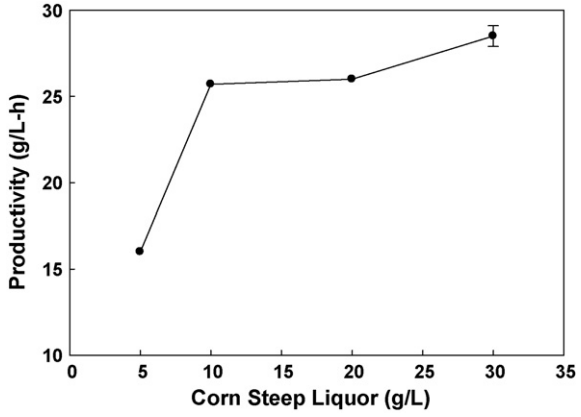

Fig. 4. Effect of corn steep liquor concentration on volumetric productivity of mannitol by *L. intermedius* NRRL B-3693 in continuous cell recycle fermentation at 37 °C, pH 5.0, and 130 rpm. Values reported are averages from duplicate experiments. Dilution rate, 0.28 h^{-1}.

L. intermedius NRRL B-3693 produced mannitol at a relatively high concentration in a low-cost medium using batch fermentation [16–18]. However, the complex nutritional requirements of LAB make them challenging for obtaining fast production rates and high volumetric productivities in such a low-cost medium. The continuous cell-recycle process showed much higher volumetric productivity, although the mannitol concentration was lower. The high productivity of the continuous process was achieved without the requirement for any expensive peptones and at lower corn steep concentrations than the fed-batch. Additionally, reducing the corn steep liquor concentration and omitting peptone resulted in a cleaner product as indicated by the lower color and absorbance at 400 nm.

A few processes have been described for mannitol production by LAB, but they had lower volumetric productivity, did not accumulate comparable amounts of mannitol, and required expensive yeast extract or peptone [3,12,15,24]. This is the first report of achieving a high productivity (40 g/(L h)) of mannitol production in any fermentation by a heterofermentative LAB.

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